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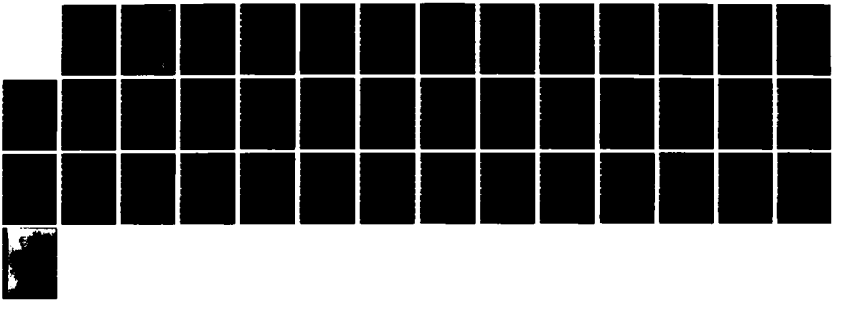

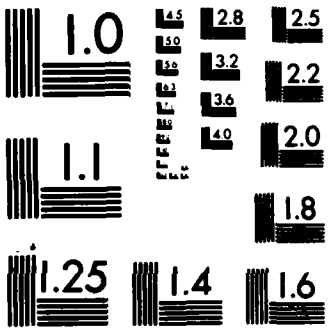

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INHIBITION OF XENOBIOTIC-DEGRADING HYDROLASES BY ORGANOPHOSPHINATES

FIRST ANNUAL PROGRESS REPORT

THOMAS M. BROWN, Principal Investigator
JAMES K. ZIMMERMAN
P. KAREN BRYSON
JOHN R. GROTHUSEN

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Organophosphinate pretreatment agents for chemical warfare defense inhibited carboxylester hydrolase from porcine liver and from rabbit liver. Recovery of rabbit liver monomeric carboxylester hydrolase to at least 30% of its initial activity was observed 48 hr. after inhibition by certain 4-nitrophenyl alkyl(phenyl)phosphinates and analogues. When ranked according to the initial rates at which their phosphinylated enzymes recovered, they were methyl(phenyl)>methyl(2-thienyl)>methyl(2-furyl)>ethyl(phenyl) di-2-

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)thienyl>diphenyl. Recovery was less than 20% in 96 hr. following inhibition by methyl(naphthyl),di-2-furyl, isopropyl(phenyl), dichloromethyl(phenyl), and bis chloromethyl substituted analogues.

High performance liquid chromatography on silica using 10% to 20% 2-propanol in hexane as mobile phase resulted in satisfactory chromatograms for all except the most polar phosphinates. This method was useful in determining purity and decomposition of the compounds.

Arylester hydrolase was purified 30-fold from rabbit serum by a sequence of polyethylene glycol fractionation, ion exchange chromatography, ammonium sulfate fractionation, molecular exclusion chromatography and pseudo-affinity chromatography. The partially purified enzyme was activated by 2.5 mM divalent calcium.

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REPORT 4

INHIBITION OF XENOBIOTIC-DEGRADING HYDROLASES BY ORGANOPHOSPHINATES

FIRST ANNUAL PROGRESS REPORT

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Summary

Carboxylester hydrolase was inhibited by various 4-nitrophenyl substituted organophosphinates; median inhibitory concentrations upon 2 min exposure were less than $6x10^{-8}$ M for 7 of 8 compounds tested. Rabbit liver monomeric carboxylesterase was completely inhibited and its spontaneous reactivation was observed following molecular size exclusion chromatography to separate the phosphinylated enzyme from inhibitor. There was little or no recovery from 5 organophosphinates nor from paraoxon. There was slow to moderate recovery from 3 other organophosphinates. Spontaneous reactivation to approximately one-half of control activity was observed following inhibition by the methyl(phenyl), methyl(2-thienyl), methyl(2-furyl), ethyl(phenyl) and di-2-thienyl substituted analogues.

Arylester hydrolase (paraoxonase) was purified 30-fold from rabbit serum in 5 days by protein precipitation and chromatography. With paraoxon as substrate, the Michaelis constant for the enzyme was 1.18 mM.

High performance liquid chromatography methods were developed for organophosphinates. Retention times of 12 organophosphinates differed from the primary decomposition product, 4-nitrophenol.

Foreword

This report contains results of the first year of a project scheduled for three years. No laboratory animals were used in the first year of this study. Citation of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

Table of Contents

1. Introduction

Organophosphinate pretreatment agents are intended to protect against chemical weapons by transient inhibition of the target enzyme acetylcholinesterase (E.C. 3.1.1.7). Several organophosphinates are rapid inhibitors of acetylcholinesterase with bimolecular reaction constants of $3x10^4$ (1).

As stated in the original proposal, the primary objective of this project is to examine the inhibition of three xenobiotic-degrading hydrolases by a variety of organophosphinates. Quantitation of the interactions of organophosphinates with sites other than the target will be necessary in order to identify candidate pretreatment agents which are least likely to produce side effects. Also, it is possible that reversible interaction with drug metabolizing enzymes could result in desirable protection in the same manner as the primary target is protected.

During the first year of the project, studies have commenced with carboxylester hydrolase (E.C. 3.1.1.1) and arylester hydrolase (E.C. 3.1.1.2) while studies of the third enzyme, fluorohydrolase (E.C. 3.8.2.1) were scheduled to begin in the second year. We report results of inhibition of carboxylesterase by organophosphinates and the spontaneous reactivation of this enzyme following complete inhibition. A purification scheme was designed for arylesterase and it is described.

The effect of organophosphinates on metabolism of xenobiotics was a subordinate objective to be studied in the second year. This work will begin next year with xenobiotics degraded by carboxylesterase.

The second subordinate objective was to determine relative toxicodynamics of enantiomers of organophosphinates following purification from racemic mixtures. We report the results of conventional high performance liquid chromatography of organophosphinates completed to prepare for purification studies. The first purification of enantiomers from a racemic mixture of 4-nitrophenyl ethyl(phenyl)phosphinate using a novel chiral stationary phase is described also.

2. Carboxylester Hydrolase Inhibition by Organophosphinates

2a. Methods:

Porcine liver carboxylesterase from Sigma Chemical Company (1 ug/ml) was incubated with inhibitor in the absence of substrate in 0.1 M sodium phosphate buffer, pH 7.5 at 37°. At intervals from 1 to 5 min after adding inhibitor, aliquots were taken for 260-fold dilution into substrate solution to measure the hydrolase activity remaining. When 1-naphthyl acetate was used as the hydrolase substrate, an apparent K_M of 0.0356 mM was computed from an Eadie-Hofstee plot. Enzymic hydrolysis of 0.35 mM 1-naphthyl acetate in 2.6 ml sodium phosphate buffer was terminated after 10 min by adding 0.5 ml 8.4 mM tetrazotized o-dianisodine in 0.425 M aqueous sodium dodecyl sulfate. The absorbance of blue color, which developed in 10 min, was measured at 600 nm against a reagent blank. Typical hydrolase activity in the control with acetone carrier added only was 0.29 mmoles min^{-1} mg $protein^{-1}$.

The organophosphinates used in these experiments were considered as four series of analogous compounds (Table 1). They were synthesized by Ash-Stevens, Incorporated, Detroit, Michigan and provided to us by C. N. Lieske of the U.S. Army Medical Research Institute for Chemical Defense, Aberdeen Proving Ground, Maryland.

Table 1. Four series of organophosphinates used in studies of inhibition of xenobiotic-degrading hydrolases.

Compound	Number	Molecular Weight
Phenyl series:		
4-nitrophenyl methyl(phenyl)phosphinate	I	277
4-nitrophenyl ethyl(phenyl)phosphinate	II	291
4-nitrophenyl isopropyl(phenyl)phosphinate	III	305
4-nitrophenyl diphenylphosphinate	IV	339
Methyl series:		
4-nitrophenyl dimethylphosphinate	V	215
4-nitrophenyl methyl(2-furyl)phosphinate	VI	267
4-nitrophenyl methyl(2-thienyl)phosphinate	VII	283
4-nitrophenyl methyl(phenyl)phosphinate	(I)	277
4-nitrophenyl methyl(1-naphthyl)phosphinate	VIII	327
Heterocycle series:		
4-nitrophenyl methyl(2-furyl)phosphinate	(VI)	267
4-nitrophenyl di-2-furylphosphinate	IX	335
4-nitrophenyl methyl(2-thienyl)phosphinate	(VII)	283
4-nitrophenyl di-2-thienylphosphinate	X	351
Halogen series:		
4-nitrophenyl bis-chloromethylphosphinate	XI	284
4-nitrophenyl monochloromethyl(phenyl)phosphinate	XII	312
4-nitrophenyl dichloromethyl(phenyl)phosphinate	XIII	347
4-nitrophenyl trichloromethyl(phenyl)phosphinate	XIV	382
4-nitrophenyl methyl(trifluoromethylphenyl)phosphinate	XV	345

2b. Results:

Preliminary experiments indicated less than 3% of control hydrolase activity remained after 5 min inhibition at $1x10^{-6}$ M organophosphinate; the isopropyl derivative was an exception as 29% of hydrolase activity remained (Table 2).

Table 2. Total inhibition of porcine liver carboxylesterase by 4-nitrophenyl organophosphinates of the phenyl series.

4-nitrophenyl alkyl(phenyl)phosphinate	% activity remaining after 5 min at $1x10^{-5}$M	$1x10^{-6}$M	$1x10^{-7}$M	$1x10^{-8}$M
methyl-	0	1	19	67
ethyl-	0	0	5	40
isopropyl-	3	29	64	91
phenyl-	0	2	7	45
paraoxon standard	-	-	4	77

Activities then were determined following 1 min intervals to 5 min of inhibition at concentrations which were not completely inhibitory (Table 3). Inhibition progressed with time but most plots of log % activity vs. time failed to pass through 100% at 0 time and the lines curved rather than progressing toward complete inhibition. Most inhibitors acted very rapidly so that no activity remained at 1 min unless the inhibitor concentration was reduced to near that of the enzyme which was $1.47x10^{-8}$ M (subunit-equivalent concentration). Due to the lack of first-order inhibition kinetics, k_i values could not be determined. Provisional I_{50} values were determined by plotting % activity remaining after 2 min versus log inhibitor concentration using the average of 2 replications at each of 3 or 4 inhibitor concentrations.

Table 3. Partial inhibition of porcine liver carboxylesterase by organophosphinates as determined at 1 min intervals of incubation.

Compound	Concentration, M	% activity remaining at				
		1	2	3	4	5 min
I	4×10^{-8}	66	67	64	52	56
	5.66	48	34	28	23	19
	8	44	29	22	18	18
II	4×10^{-8}	83	71	56	40	47
	5.66	63	46	39	30	24
	8	41	19	12	8	6
III	$*0.5\times10^{-6}$	85	63	48	46	45
	1	60	48	44	31	36
	2	48	63	35	31	31
	*2.83	42	22	22	16	11
	4	28	18	14	11	9
	*8	17	8	4	1	0
IV	$*1.41\times10^{-8}$	78	73	77	72	70
	*2	46	36	39	10	14
	2.83	55	41	27	16	14
	4	30	10	8	6	5
	*8	15	5	1	1	0
XII	$*0.8\times10^{-8}$	90	87	87	72	79
	5.66	39	26	20	17	15
	8	32	24	20	14	16
VI	1.42×10^{-8}	79	66	64	61	65
	2.83	59	43	45	46	48
	5.66	45	32	30	26	27
	$*8\times10^{-7}$	9	11	11	11	13
VII	1.42×10^{-8}	67	56	49	44	37
	2.83	49	33	23	18	16
	5.66_{-7}	27	13	10	4	3
	8×10^{-7}	0	0	0	0	0
X	0.708×10^{-8}	70	60	56	55	56
	1.42	40	24	19	13	11
	2.83	23	10	7	5	3
	5.66	9	5	4	3	2
paraoxon standard	8×10^{-8}	45	22	24	17	12

*One experiment only at this concentration; at all other concentrations means for duplicate observations on different days are given.

Three heterocyclic organophosphinates and five phenylphosphinates were inhibitors of carboxylesterase (Table 4). By this estimate, 4-nitrophenyl di-2-thienylphosphinate was most inhibitory of eight compounds tested; 4-nitrophenyl isopropyl(phenyl)phosphinate was least inhibitory. Six other compounds within these series were similar in potency having median inhibitory values within a 3-fold range of concentrations.

Replacing the phenyl moiety with the thienyl group resulted in 3-fold increases in inhibition (Table 4). This was observed in both the methyl (2-thienyl) compound and the di-2-thienyl compound when compared to their phenyl analogues.

Table 4. Inhibition of porcine liver carboxylesterase by organophosphinates as estimated by median inhibitory concentration for 2 min incubation.

Inhibitor	I_{50} (2 min), M*
Heterocycle series:	
4-nitrophenyl di-2-thienylphosphinate	7.4×10^{-9}
4-nitrophenyl methyl(2-thienyl)phosphinate	1.7×10^{-8}
4-nitrophenyl methyl(2-furyl)phosphinate	2.5×10^{-8}
Phenyl series:	
4-nitrophenyl diphenylphosphinate	2.1×10^{-8}
4-nitrophenyl chloromethyl(phenyl)phosphinate	2.8×10^{-8}
4-nitrophenyl methyl(phenyl)phosphinate	5.1×10^{-8}
4-nitrophenyl ethyl(phenyl)phosphinate	5.3×10^{-8}
4-nitrophenyl isopropyl(phenyl)phosphinate	9.6×10^{-7}

*Values are provisional estimates since a 10-fold excess of inhibitor over enzyme concentration could not be achieved; see text.

In these experiments, enzyme and inhibitor were incubated first in the absence of substrate; then the inhibition mixture was diluted into substrate solution to measure the enzyme activity remaining. The substrate used to assess the residual activity had no effect on the results as observed by substituting either phenylthioacetate or phenylthiobutyrate for 1-naphthyl acetate. Thiols produced by hydrolysis of these alternative substrates were measured upon a chromogenic reaction (2).

When 4-nitrophenyl di-2-thienylphosphinate was the inhibitor and phenylthiobutyrate the subsequent substrate, a linear relationship was observed between log % activity and inhibition time; however, the line did not pass through 100% (Table 5). These results were very similar to those obtained with 1-naphthyl acetate as substrate (Table 3).

Table 5. Inhibition of porcine liver carboxylesterase by 1.42×10^{-8} M 4-nitrophenyl di-2-thienylphosphinate with residual activity assessed by hydrolysis of phenylthiobutyrate.

Inhibition time, min	% activity remaining*
1	45.2
2	29.6
3	30.7
4	19.2
5	17.6

*Average of duplicate observations on different days.

2c. Discussion:

In order to obtain bimolecular reaction constants, i.e. k_i values, by which inhibitory power of various organophosphinates against carboxylesterase can be compared, the experiments described above must produce linear plots of inhibition time versus log % activity and the lines must pass through 100% activity at 0 time (3).
Since our results typically were curved lines not passing through 100% activity, only provisional median inhibitory concentrations can be obtained.

From the provisional median inhibitory concentrations in Table 4, k_i can be estimated from the formula:

$$k_i = \frac{\ln \frac{v_o}{v}}{I_{50} t}$$

If it is assumed that there was 100% activity at a 0 time (v_o), and since the I_{50} was determined using activity at 2 min, then:

$$k_i = \frac{\ln \frac{100}{50}}{I_{50}{}^{x2}} = \frac{.693}{I_{50}{}^{x2}}$$

Bimolecular reaction constants estimated by this method for the compounds tested (Table 4) range from 3.61×10^5 for 4-nitrophenyl isopropyl(phenyl)-phosphinate to 4.68×10^7 for 4-nitrophenyl di-2-thienylphosphinate.

Our results indicate that 4-nitrophenyl organophosphinates of the heterocycle and phenyl series are very rapid inhibitors of carboxyl-esterase; estimated k_i values are conservatively less than actual values. With 4-nitrophenyl dialkylphosphinates against carboxylesterase, k_i values were 1×10^6 for the dipropyl and diheptyl analogues and 2.2×10^6 for the

diethyl analogue which was 600-fold greater than the k_i against acetylcholinesterase (4).

The curvature in the lines obtained from results for most of these phosphinates is expected since the calculated enzyme concentration is 1.4×10^{-8} M and most inhibitors were used at approximately an equivalent concentration rather than the 10-fold excess necessary to obtain linear plots (5). This problem cannot be overcome in our method since a 10-fold excess resulted in nearly total inhibition.

Development of a more satisfactory method for comparing these carboxylesterase inhibitors is in progress. Using different substrates and a controlled thermoelectric flowcell for the spectrophotometer, inhibitors will be added to the enzyme-substrate mixture; the change in an established substrate-hydrolysis progress curve will be used to assess inhibition rates. Phenylthiobutyrate will be used as substrate for this procedure. Ethylthioacetate and ethylthioproprionate have been purchased and ethylthiobutyrate has been synthesized to provide additional candidate substrates.

3. Recovery of Phosphinylated Carboxylester Hydrolase

Since inhibition by organophosphinates was very rapid but difficult to assess quantitatively, experiments were initiated to examine the recovery of the inhibited enzyme when separated from excess inhibitor. Rabbit liver monomeric carboxylesterase, purified and crystalline, was provided for these experiments by Dr. A.R. Main, North Carolina State University, who was a consultant to this project.

3a. Methods:

Spontaneous reactivation experiments were similar to those performed with phosphinylated acetylcholinesterase (6). A molecular size exclusion column 10.8 cm by 0.8 cm was prepared from 1 g Sephadex G-25-150, swollen 3 h in 0.1 M sodium phosphate buffer, pH 7.6, and calibrated with Blue Dextran and N-2, 4-dinitrophenyl-L-phenylalanine. Rabbit liver monomeric carboxylesterase (7) at 0.1 mg/ml was inhibited at room temperature for 3 min with 4.87×10^{-4} M phosphinate added in acetonitrile (1% final concentration). It was necessary to use 9.74×10^{-4} M of the 2-furyl derivatives, VI and IX, for 30 min to inhibit >90% of the enzyme.

The phosphinylated enzyme was separated from inhibitor by adding 0.5 ml of the mixture to the column and collecting the initial 4 ml eluate flowing at 0.5 ml/min. The eluate was diluted to 50 ml and held at 37° in a shaking water bath. Recovery of enzyme activity from the column was 97.1% in this initial eluate.

Spontaneous reactivation was assessed by adding 20 µl of the purified, phosphinylated enzyme to 2.6 ml 0.2 mM 1-naphthyl butyrate for 10 min at 37°, then terminating the reaction with tetraazotized o-dianisidine and measuring the absorbance as in Section 2a. To examine reactivation at 25°, the diluted eluate from the column was divided and a subsample was held in

a second water bath. This sample was assayed for activity at 25° and compared to a companion control. Inhibition and reactivation was repeated for each phosphinate on 4 days.

3b. Results:

Carboxylesterase recovered more than 80% of control activity after complete inhibition by compounds I, II and IV of the phenyl series (Fig. 1). Initially, spontaneous reactivation was extremely rapid from the methyl(phenyl)phosphinylated carboxylesterase which recovered one-half its activity in only 4 h. Following inhibition by the phenyl series, carboxylesterase reactivation ranked as methyl>ethyl>phenyl>>isopropyl.

Reactivation was generally faster following inhibition by compounds of the methyl series (Fig. 2) than it was following the phenyl series. Recovery from I, VI and VII exceeded 25% in 4 h and it exceeded 80% in 6 days. Reactivation from VIII was very slow. Compound V was not available. Based on initial reactivation rates, recovery from the phenyl series was in the order methyl>2-thienyl>2-furyl>>1-naphthyl.

Reactivation plots of carboxylesterase from compounds VI, VII and X of the heterocycle series produced curved lines which intersected at approximately 61% recovery at 41 h (Fig. 3). Biphasic reactivation kinetics will be discussed below. There was no progressive reactivation following inhibition by IX. Based on recovery during 24 h, reactivation from compounds of the heterocycle series was ranked as methyl(2-thienyl)->methyl(2-furyl)>di-2-thienyl>>di-2-furyl.

Following inhibition by compound XII, XIII and XIV of the halogen series, reactivation by carboxylesterase was inversely related to the number of chlorine atoms in the inhibitor (Fig. 4). While XIV did not completely inhibit carboxylesterase in the 3 min reaction, enzyme activity declined following elution from the molecular-size exclusion column; thereafter, it did not reactivate. Recovery from XI was slow and data were highly variable; XV was not tested. Recovery rates for the chloro-methyl-(phenyl)phosphinylated carboxylesterase were ranked as monochloro>dichloro->trichloro.

When all inhibitors were compared for carboxylesterase reactivation in 24 h, those from which recovery was greatest contained both one alkyl substituent smaller than isopropyl and one aryl or heteroaryl substituent smaller than naphthyl (Table 6). The exception was X which contained two thienyl groups. Compounds can be grouped according to the rates at which recovery proceeded as follows:

fast recovery: I, II, VI, VII, and X;
moderate recovery: IV and XII;
slow progressive recovery: XIII;
no progressive recovery: III, VIII, IX, XI, XIV and paraoxon.

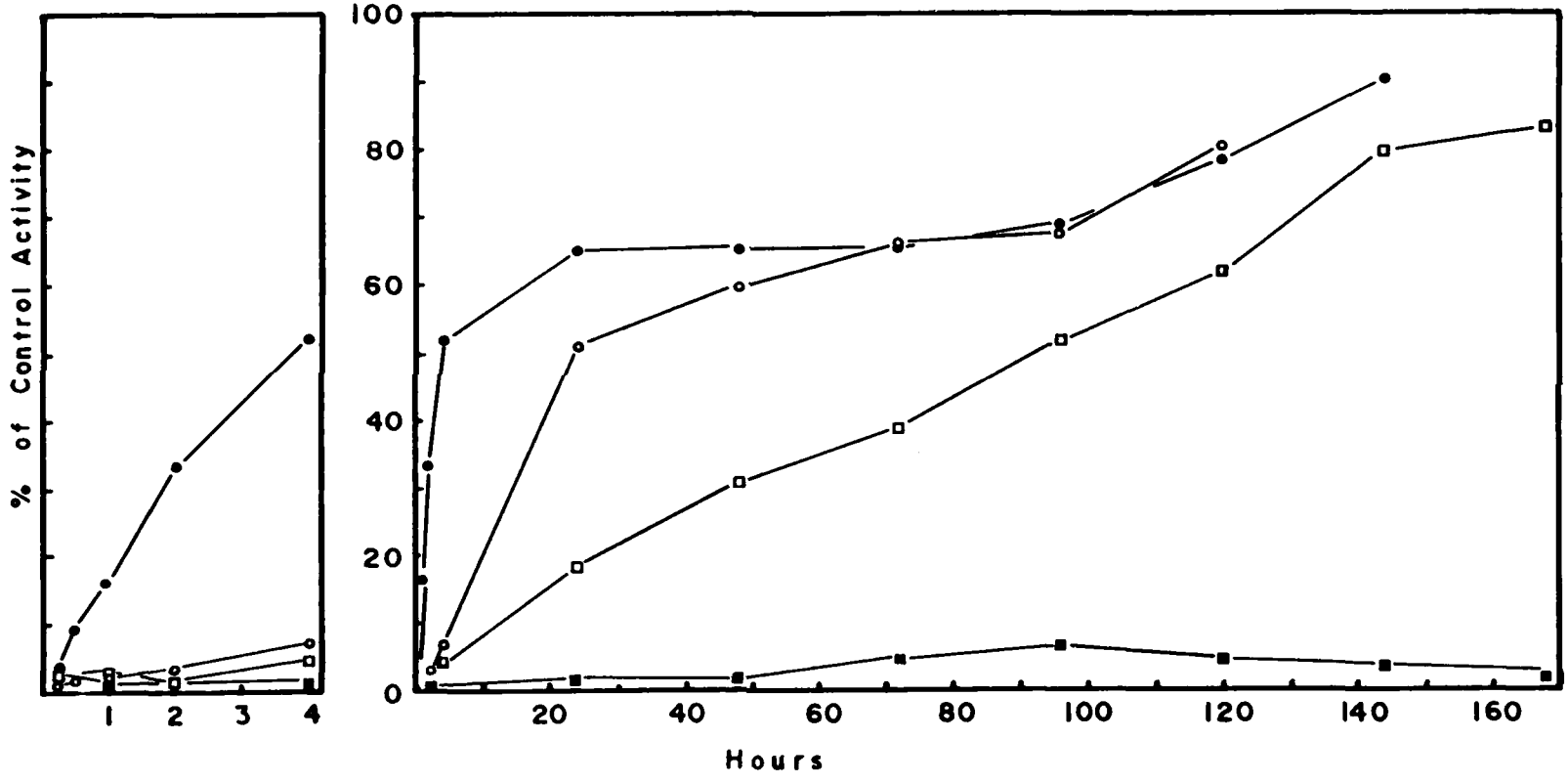


Figure 1. Spontaneous reactivation at 37° of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the phenyl series; closed circle is methyl(phenyl), open circle is ethyl(phenyl), closed square is isopropyl(phenyl), and open square is diphenyl.

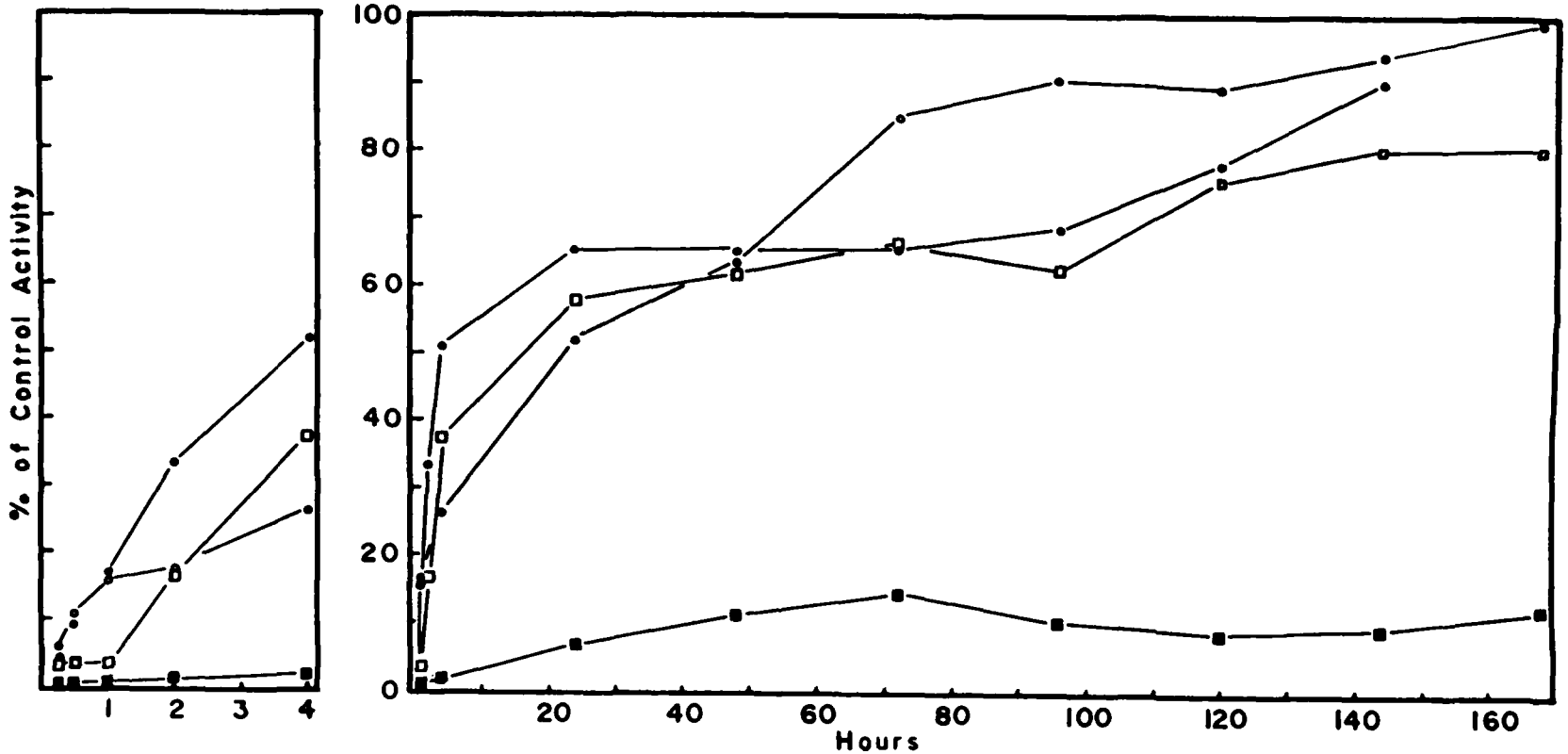


Figure 2. Spontaneous reactivation at 37° of rabbit liver monomeric carboxylester hydrolase following inhibition of 4-nitrophenyl substituted organophosphinates of the methyl series; closed circle is methyl(phenyl), open circle is methyl (2-furyl), closed square is methyl(naphthyl), and open square is methyl(2-thienyl).

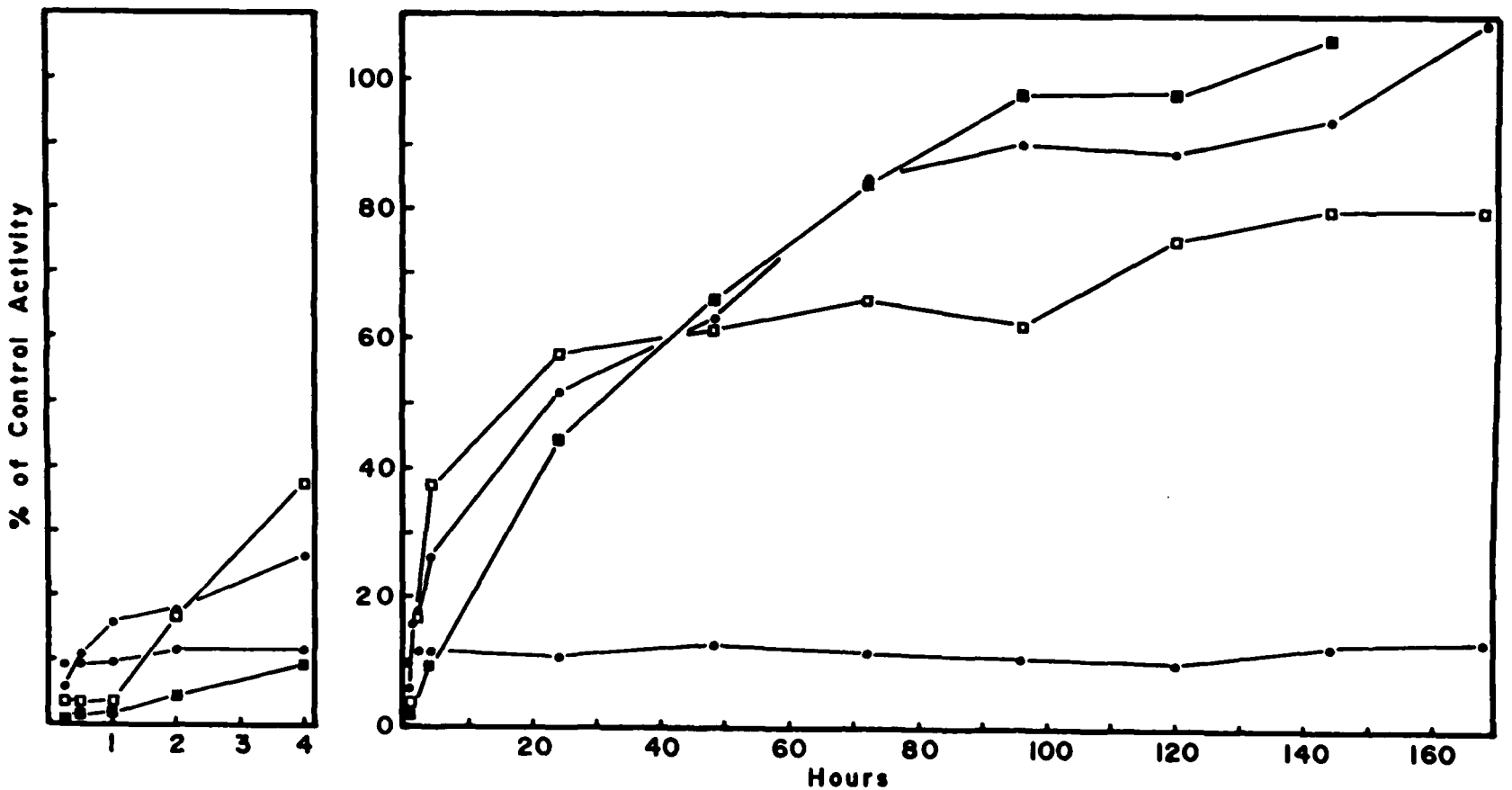


Figure 3. Spontaneous reactivation of 37° of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the heterocycle series; closed circle is methyl(2-furyl), open circle is di-2-furyl, closed square is di-2-thienyl, and open square is methyl(2-thienyl).

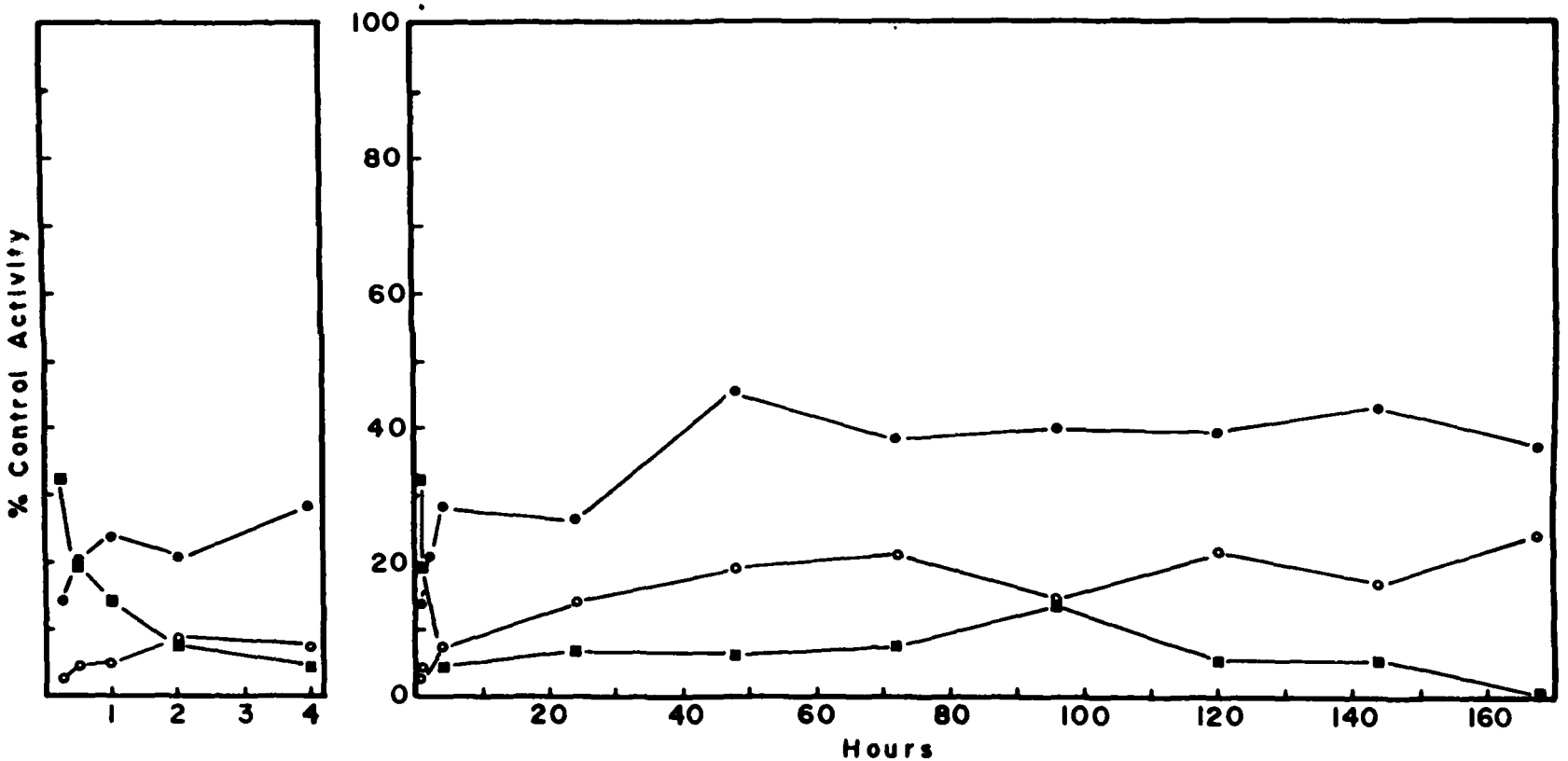


Figure 4. Spontaneous reactivation at 37° of rabbit liver monomeric carboxylester hydrolase following inhibition by 4-nitrophenyl substituted organophosphinates of the halogen series; closed circle is monochloromethyl(phenyl), open circle is dichloromethyl(phenyl), and closed square is trichloromethyl(phenyl).

Table 6. Spontaneous reactivation of carboxylester hydrolase following inhibition by substituted 4-nitrophenyl organophosphinates.

Compound	Substitutions on phosphinate	Reactivation, % of control activity ± s.e. 24 h	72 h
I	methyl(phenyl)	65.3 ± 5.6	65.6 ± 4.8
VII	methyl(2-thienyl)	57.6 ± 2.8	66.0 ± 2.5
VI	methyl(2-furyl)	51.9 ± 4.9	85.1 ± 2.1
II	ethyl(phenyl)	50.8 ± 1.9	65.8 ± 2.6
X	di-2-thienyl	44.4 ± 8.2	84.0 ± 9.1
XII	monochloromethyl(phenyl)	26.5 ± 2.2	40.2 ± 14.6
VI	diphenyl	18.5 ± 3.9	39.0 ± 3.0
XIII	dichloromethyl(phenyl)	14.2 ± 3.5	21.4 ± 3.5
XI	bis-chloromethyl	12.4 ± 4.6	13.6 ± 9.4
IX	di-2-furyl	10.6 ± 1.3	11.4 ± 1.0
VIII	methyl(1-naphthyl)	6.8 ± 0.4	14.7 ± 4.7
XIV	trichloromethyl(phenyl)	6.5 ± 2.0	7.5 ± 5.2
-	paraoxon standard	5.9 ± 2.1	5.4 ± 3.6
III	isopropyl(phenyl)	1.5 ± 0.9	4.4 ± 1.0

3c. Discussion:

Reactivation of carboxylesterase did not appear to follow first order kinetics with the exception of recovery from X (Fig. 3). Biphasic reactivation was observed following inhibition by I, II (Fig. 1) and VII (Fig. 3) which are chiral and assumed to be composed of a racemic mixture of + and - enantiomers. Of the three achiral organophosphinates tested, IV produced reactivation at a constant rate (Fig. 1), X produced approximate first-order kinetics (Fig. 3), while there was no significant reactivation from IX.

The rate of recovery from chiral VI was equal that from I for 1 h and then slowed to approximate first order kinetics after 4 h (Fig. 2). Biphasic kinetics were most apparent from I, II and VII since the initial rapid phase ended at approximately 50% recovery.

Biphasic reactivation kinetics could result from at least two different mechanisms; (i) presence of two enantiomeric forms of the phosphinylated enzyme which recover at different rates and (ii) inhibition and reactivation at two different sites on the enzyme. The former mechanism could produce an inflection at 50% if the inhibition were due to both enantiomers of the phosphinate; however, this was not observed with methyl(phenyl)phosphinylated acetylcholinesterase at 25° (6). The latter possibility must be considered also since carboxylesterases have exhibited non-Michaelis-Menten kinetics which have been explained using modifier-site schemes (8).

At 25°, reactivation rates from I and II were reduced from the rates at 37° (Table 7). Since 1-naphthyl butyrate and other carboxylesterase substrates are limited in solubility, the remaining reactivation studies were performed at 37°.

Table 7. Effect of temperature on spontaneous reactivation of carboxylester hydrolase following inhibition by organophosphinates.

Inhibitor	Time, h	Reactivation, % of control activity		
		25°	37°	25°/37°
I	4	21.7	51.8	0.42
I	24	46.5	65.3	0.71
II	4	1.9	6.9	0.27
II	24	26.1	50.8	0.51

4. Design of Arylester Hydrolase (Paraoxonase) Purification

4a. Methods:

Arylester hydrolase was purified from rabbit serum by precipitation and chromatography in which the fractions retained were those which hydrolyzed paraoxon. Paraoxon, 4-nitrophenyl diethylphosphate, was obtained from Sigma Chemical Company. Routine assays for activity were performed by adding 0.2 ml 3mM paraoxon in pH 7.5, 0.1 M sodium phosphate buffer with 0.01% Triton X-100 to a 25-100 µl aliquot of the sample. Activity was assessed visually by the yellow color of the 4-nitrophenol produced by hydrolysis in 10 min.

Approximately two liters of rabbit blood were obtained freshly from a slaughterhouse and allowed to clot at room temperature for two hours in a glass jar. The resulting mixture was filtered through cheesecloth and the clot gently squeezed. The filtrate was centrifuged for 10 min at 677 x gravity in a Sorvall RC-5 centrifuge. The reddish-orange supernatant was retained as serum.

To precipitate protein from serum, 12 g solid PEG 4000 (polyethyleneglycol) was added per 100 ml of serum. After an hour of stirring at 4° the mixture was centrifuged at 12,062 x gravity for 30 min. The volume of the supernatant was measured and 6 g of PEG-4000 were added per 100 ml; this mixture was stirred and centrifuged as above. The red supernatant was discarded and the tan pellet gently resuspended in 10 mM sodium phosphate buffer, pH 6.7. A 2.5x30 cm column of DEAE Sepharose CL-6B was prepared and equilibrated in the same sodium phosphate buffer and then resuspended precipitate was applied.

The sample was eluted from the DEAE column by a linear gradient of increasing ionic strength supplied directly from a mixing chamber originally containing 400 ml of buffer into which flowed buffer plus 0.4 M sodium chloride from a second chamber containing 400 ml. A red band passed through in the void volume, and the pink, orange, yellow and blue bands were eluted. Paraoxonase activity was located within, or directly behind, the yellow band and it extended into the blue fraction. Activity was located within the second of two very broad protein peaks detected by monitoring elution at 280 nm (Fig. 5).

Active fractions were pooled and saturated ammonium sulfate solution, pH 7.0, was added to 45% of saturation. This mixture was stirred for 2 h, then centrifuged for 60 min at 48,246 x gravity. The supernatant was collected and brought to 55% ammonium sulfate saturation as above, then it was stirred overnight. Following centrifugation for 60 min at 48,246 x gravity, the clear supernatant was discarded and the light blue to white precipitate was resuspended in 10 mM sodium phosphate buffer, pH 6.7, containing 2.5 mM calcium chloride and 100 mM sodium chloride (buffer A).

The resuspended 45-55% ammonium sulfate fraction was applied to a 2.5x80 cm column of polyacrylamide-agarose AcA34 (LKB Instruments, Inc., Rockville, Maryland) equilibrated in buffer A. Elution with buffer A resulted in resolution of paraoxonase between two major contaminating peaks of protein (Fig. 6).

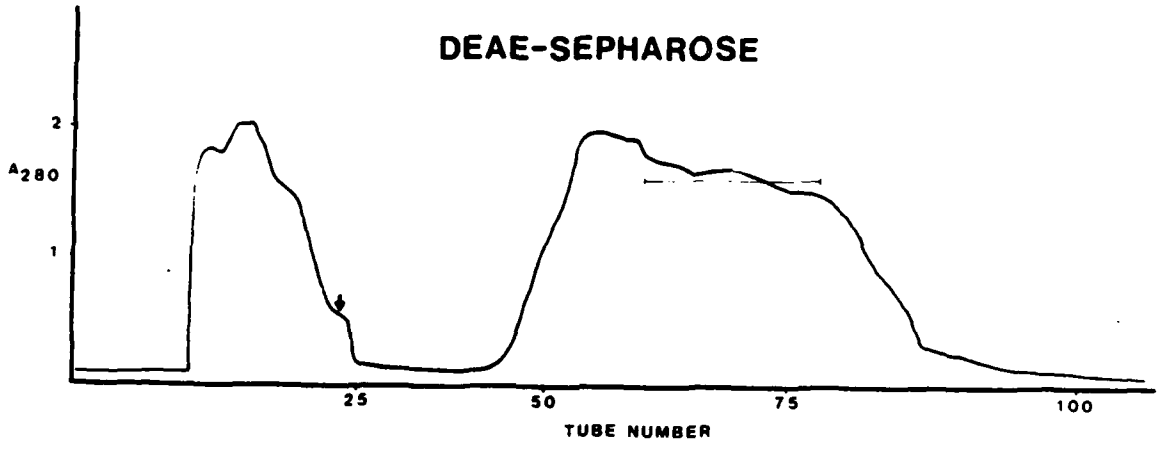


Figure 5. Ion exchange chromatography in purification of rabbit serum arylester hydrolase; arrow marks beginning of sodium chloride gradient; horizontal bar represents fraction hydrolyzing paraoxon.

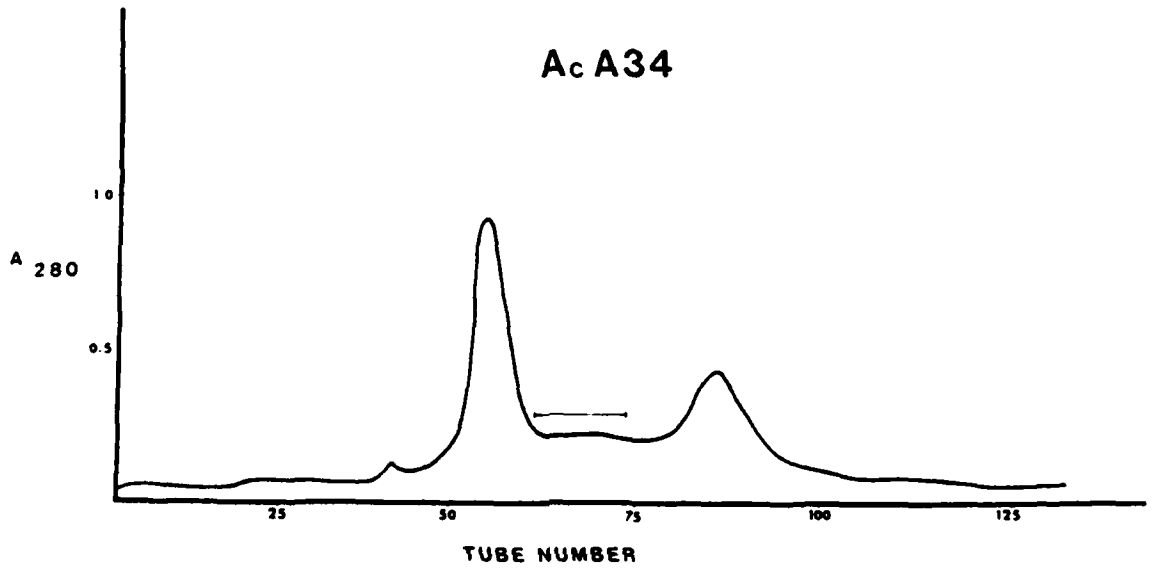


Figure 6. Polyacrylamide-agarose molecular size exclusion chromatography in purification of rabbit serum arylester hydrolase; horizontal bar marks fractions hydrolyzing paraoxon.

Paraoxonase fractions were pooled and applied to a column of dye-ligand Matrex Gel Red A® (Amicon Corporation, Danvers, Massachusetts) equibrated with buffer A. Paraoxonase activity passed through without binding while impurities were bound to the column during elution with buffer A (Fig. 7).

4b. Results:

Paraoxonase was purified 30-fold from rabbit serum so that the final specific activity was 175 nmoles 4-nitrophenyl produced/min/mg of protein (Table 8). The procedure was completed in 5 days.

The Michaelis constant, K_M, for the purified enzyme with 0.18 mM calcium chloride was 1.18 mM as determined from a weighted Lineweaver-Burk plot (Fig. 8). The average reported K_M of rabbit serum paraoxonase without purification was 1.39 mM (9).

After Matrex Gel Red A chromatography, paraoxonase excited at 285 nm emitted a peak of fluorescence at 350 nm (Fig. 9) and it absorbed ultraviolet light with a 280/260 ratio of 1.3. In a previous purification of paraoxonase from sheep serum, different spectroscopic results were obtained for the purified enzyme which emitted blue fluorescence and absorbed with a lower 280/260 nm ratio of 0.85 (10). The 385-fold purification from sheep serum resulted in a purified paraoxonase with a K of 4.2 mM as opposed to 0.29 mM in the serum; final specific activity was 1.0 μmole 4-nitrophenol/min/mg.

Decrease in K_M with purification of sheep serum (10) and loss of paraoxonase activity upon EDTA addition to rabbit serum (11) suggested the loss of an activator. Several reports have mentioned divalent calcium as an activator of arylester hydrolase (vide review by 12). When the DEAE-agarose chromatographed paraoxonase fraction was incubated with calcium chloride at concentrations from 1 to 10 mM and subsequently diluted for assay at 0.0189 to 0.189 mM, the optimum calcium concentration in the assay was found to be 0.0472 mM.

4c. Discussion:

Several parts of the purification method will be examined for improvement. The major loss of activity during ammonium sulfate fractionation of rabbit paraoxonase was not encountered during similar procedures with sheep paraoxonase (10). The activity which was lost was not found in the 45% precipitate nor in the 55% supernatant. Modifications will include increasing pH of saturated ammonium sulfate to 7.6 and maintaining 2.5 mM calcium.

Other co-factors may be involved other than calcium. Loss of activity could be due to loss of a small protein co-factor. Many membrane-associated enzymes are dependent on both divalent calcium and the small protein, calmodulin (13).

A more efficient final purification step would result if paraoxonase were bound to an affinity column so that selective elution gradients could be developed. With Matrex Gel Red A, activity was not bound. Other columns used without success include thiol-agarose, hydroxyapatite, and an

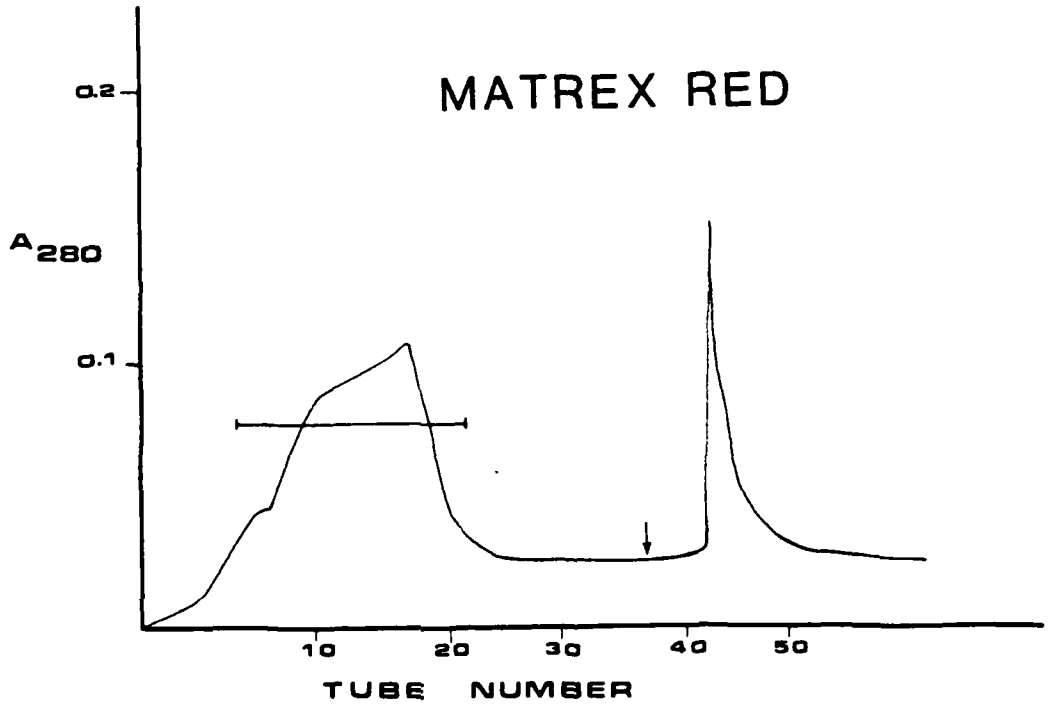


Figure 7. Dye-ligand affinity chromotography in purification of arylester hydrolase; horizontal bar represents fractions hydrolyzing paraoxon; arrow marks beginning of elution with 1.0 M sodium chloride in buffer.

Table 8. Purification of arylester hydrolase (paraoxonase) from rabbit serum.

Step	Volume (ml)	Protein Concentration[a] (mg/ml)	Total Protein (mg)	Total Units[b]	Specific Activity (units/mg)	Purification	Yield (%)
Serum	2140	59.2	127,000	738.2	0.0059	1.0	100
12% PEG precipitate	2220	40.8	90,600	696.5	0.0077	1.31	94.3
12-18% PEG cut	25	216	5,410	179.8	0.0333	5.69	24.3
DEAE Sepharose	184	14.2	2,590	121.3	0.0469	8.02	16.4
45-55% Ammonium Sulfate cut	5	ND[c]	---	5.39	---	---	---
AcA34	108	0.57	61.3	7.07	0.115	19.3	0.96
Matrex Red	113	0.22	24.8	4.33	0.175	29.8	0.59

[a] By the biuret method using bovine serum albumin as a standard.

[b] μmoles of 4-nitrophenol produced per minute from paraoxon.

[c] Not determined because of the effect of NH_3 on biuret reaction.

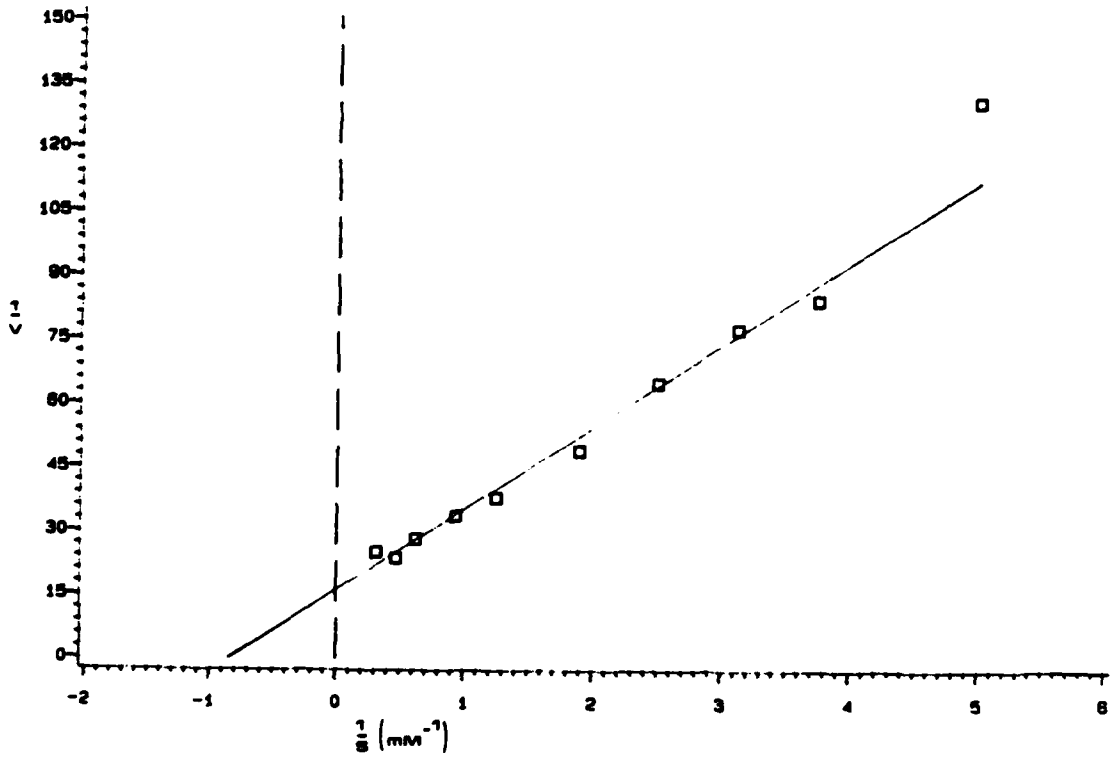


Figure 8. Weighted Lineweaver-Burk plot of arylester hydrolase purified 30-fold from rabbit serum, substrate was paraoxon.

Figure 9. Fluorescence emission spectrum of 30-fold purified rabbit serum arylester hydrolase upon excitation at 285 nm.

affinity column prepared from 4-aminophenylpinacolyl methylphosphonate hydrocloride, a competitive inhibitor of rabbit serum paraoxonase (9).

5. High Performance Liquid Chromatography (HPLC) of Organophosphinates:

5a. Methods:

Organophosphinates were examined for chromatographic properties on a 10 µm silica gel HPLC column 0.4 x 25 cm (Whatman Partisil PXS 10/25) enclosed in a water jacket held at 36° by a Neslab circulator. A 0.4 x 10 cm guard column packed with 40 m silica (Whatman Pellosil) preceded the analytical column.

The instrument consisted pf a Tracor 980A solvent programmer, Tracor 950 high-pressure pump, Valco injector with 10 µl loop, Tracor 970A ultraviolet/visible detector, and Fisher recorder. Solvents were mixed in the programmer and then pumped isocratically at 800-1000 psi through the column and detector at 1.0 ml/min. Detection was by absorbance at 270 nm to produce a signal at 0.08 optical density units full scale which was attenuated as needed to 0.32 o.d.u.f.s. The recorder was operated at 1 mV full scale and 0.333 cm/min.

Samples of organophosphinates were dissolved in acetonitrile (HPLC grade, Fisher) and then diluted 1:19 with 20% 2-propanol in hexane (distilled-in-glass grade, Burdick and Jackson). Approximately 2 µg were injected. The linear range was > 20-fold, and limit of detection was < 20 ng for II. Retention times were measured from the time of injection.

5b. Results:

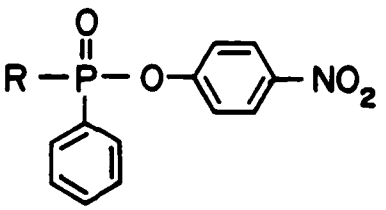
Retention time of organophosphinates in the phenyl series was directly related to polarity (Table 9). For comparison, 4-nitrophenol and paraoxon were retained 9.8 and 12.8 min, respectively. In the halogenated series (Table 10), retention time was inversely related to the number of chlorine substitutions of the methyl group. Two diheterocycle-substituted derivatives were retained longer than was 4-nitrophenyl diphenylphosphinate (Table 11).

Acceptable chromatography of phosphinates in the methyl series required the increased elutropic power provided by 20% 2-propanol in hexane (Table 12). The naphthyl derivative, which was theoretically least polar, was retained least of this series. In this solvent mixture, 4-nitrophenol and paraoxon were retained 5.5 and 8.2 min, respectively.

5c. Discussion:

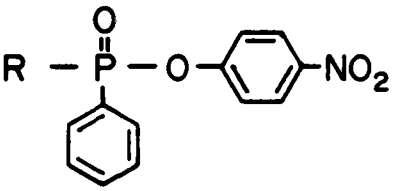
These methods will be useful for identification of organophosphinates, for determination of purity, and for assessing decomposition to 4-nitrophenol. These HPLC solvents are also recommended for use in separating enantiomers on a column of (R)-N-3,5-dinitrobenzoylphenylglycine (DNBPG) chiral stationary phase ionically bonded to 5 µm

Table 9. High performance liquid chromotography of phenyl-series 4-nitrophenyl phosphinates on silica gel at 36° with 10% 2-propanol in hexane.



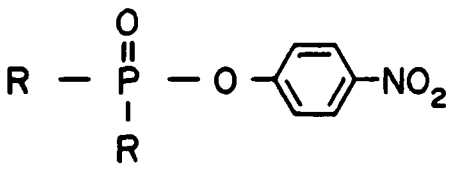
R	Retention time, min (sd)	n
CH_3-	>30	-
CH_3CH_2-	15.5 (2.0)	8
$CH_3CH(CH_3)-$	9.3 (0.7)	7
	8.1 (0.6)	8

Table 10. High performance liquid chromatography of halogen-series 4-nitrophenyl phosphinates on silica gel at 36° with 10% 2-propanol in hexane.



R	Retention time, min (sd)	n
CH_3-	>30	-
CH_2Cl-	15.1 (1,8)	6
$CHCl_2-$	7.7 (0.6)	7
CCl_3-	5.7 (0.3)	7

Table 11. High performance liquid chromatography of bis-substituted 4-nitrophenyl phosphinates on silica at 36° with 10% 2-propanol in hexane.



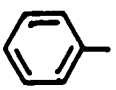
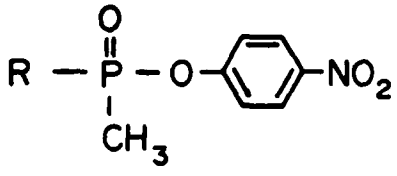
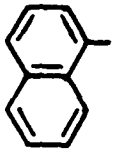
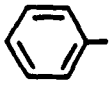
R	Retention time, min	(sd)	n
	9.7	(2.5)	12
	12.4	(2.5)	9
	13.2	(1.8)	8

Table 12. High performance liquid chromatography of methyl-series 4-nitrophenyl phosphinates on silica at 36° with 20% 2-propanol in hexane.



R	Retention time, min (sd)	n
	12.8 (2.1)	4
	18.4 (2.4)	4
	19.3 (2.3)	3

aminopropyl silica Bakerbond™ Chiral Phase, J. T. Baker Research Products, Phillipsburg, New Jersey (14).

Preliminary chromatography of II, III, IV and XIV on the DNBPG column resulted in a single peak for achiral IV, while chiral compounds II, III and XIV were partially separated into two peaks. Optimum conditions for separation of the putative enantiomers of II were mobile phase: 5% 2-propanol in n-hexane; flow rate: 1.0 ml per min; and column temperature 18 °C. Injection of 50 µl of II resulted in partial separation of the putative enantiomers in an approximate ratio of 1:1 (Fig. 10).

To obtain the lesser retained enantiomer, eluate was collected from the beginning of the peak at baseline to a point just past the first peak; for the second enantiomer, eluate was collected from declining, later side of the second peak (Fig. 10). Accumulation from 22 injections of 50 µg resulted in a yield of 0.190 mg of the first enantiomer and 0.397 mg of the second enantiomer which was less pure (Fig. 11). Identification of these enantiomers has not been confirmed.

6. Conclusions

Various 4-nitrophenyl organophosphinate pretreatment agents were rapid inhibitors of carboxylester hydrolase, a drug metabolizing enzyme found in mammalian liver microsomes and in other tissues. Phosphinylated carboxylesterases reactivated spontaneously following separation from certain inhibitors. Recovery rates varied widely depending on the chemistry of the inhibiting phosphinyl group.

It is likely that ester hydrolysis of xenobiotics would be diminished following administration of those pretreatment agents from which carboxylesterase recovered very slowly (III, VIII, IX, XI, and XIV). In the same light, it is probable that organophosphorus warfare poisons would inhibit carboxylesterase rapidly and irreversibly as did paraoxon in this study. A protective action could result from those pretreatment agents which inhibited carboxylesterase rapidly but reversibly (I, II, IV, VI, VII, X, XII, and XIII).

Biphasic reactivation kinetics following inhibition by racemic mixtures of certain chiral organophosphinates suggested stereospecific recovery. This hypothesis is under investigation as enantiomers of chiral organophosphinates are resolved by high performance liquid chromatography.

Arylester hydrolase was purified 30-fold in 5 days from rabbit serum; however, yield was very low. Investigations of organophosphinate interaction with this enzyme are in progress.

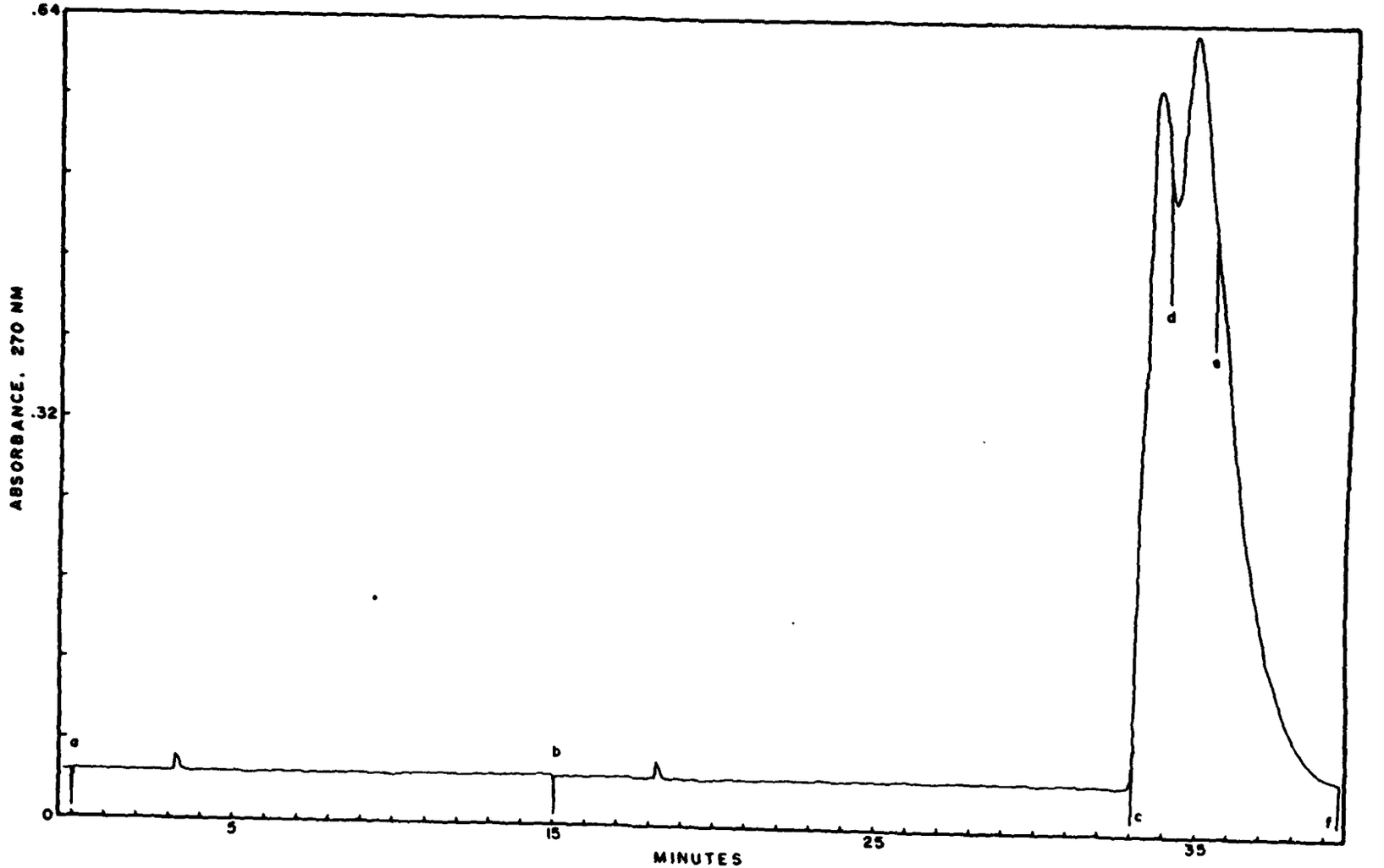


Figure 10. Chiral stationary phase high performance liquid chromatography of 4-nitrophenyl ethyl(phenyl)-phosphinate at 18° with 5% 2-propanol in hexane mobile phase; a-injected 50 µg sample, b-injected second 50 µg sample (peaks not shown), c to d - collected first peak, and 3 to f - e collected second peak. Both peaks collected were from injection a.

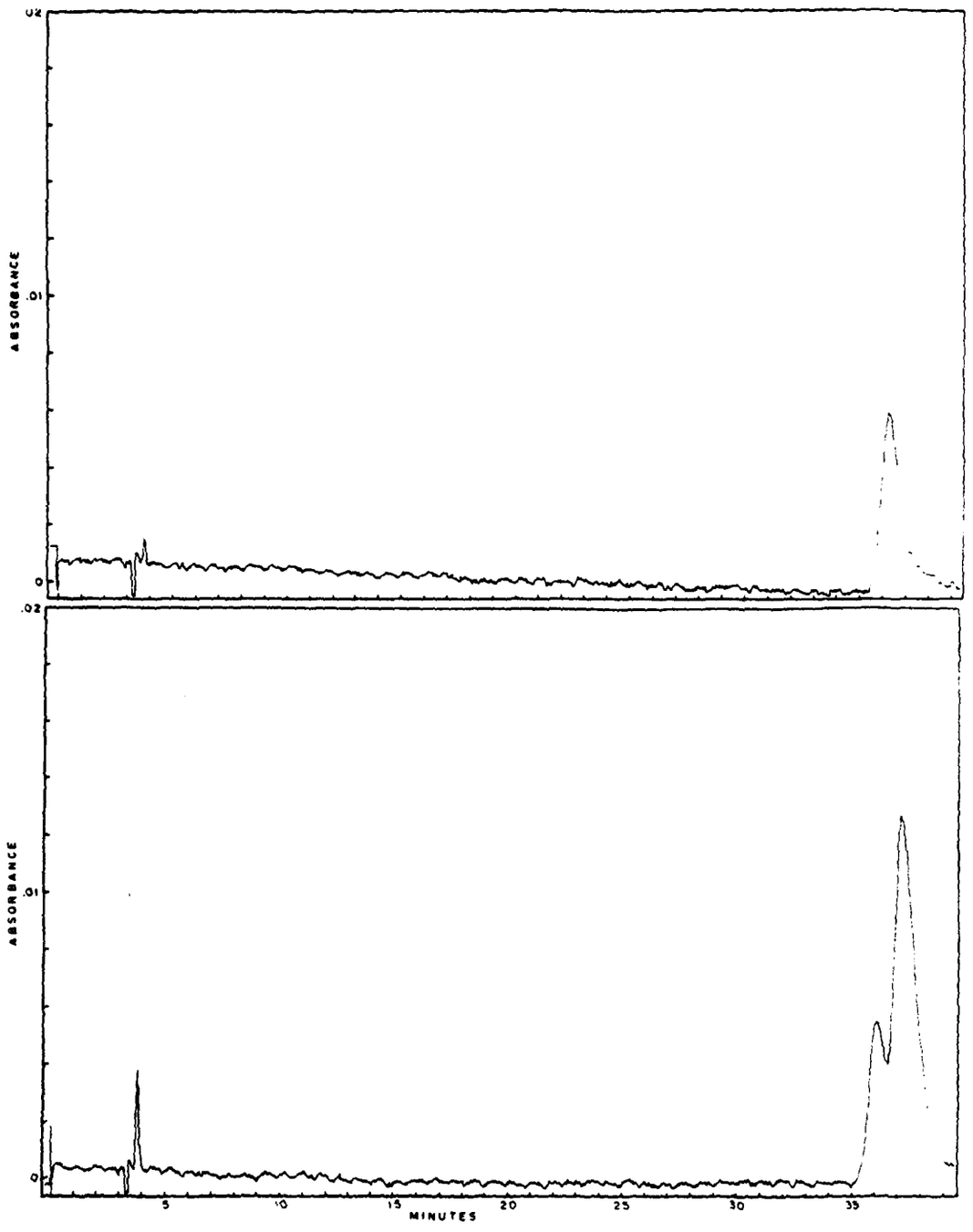


Figure 11. Chiral stationary phase high performance liquid chromatography of putative resolved enantiomers of 4-nitrophenyl ethyl(phenyl)-phosphinate; upper panel is lesser-retained enantiomer, lower panel is greater-retained enantiomer; absorbance at 270 nm.

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